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RESEARCH COMMUNICATION

Soluble low-K_m 5′-nucleotidase from electric-ray (*Torpedo marmorata*) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage

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Soluble and membrane-bound low-K_m 5′-nucleotidase was isolated from high-speed supernatants and membrane fractions derived from the electric organ of the electric ray (*Torpedo marmorata*) or from bovine brain cerebral cortex. Purification of both enzymes included chromatography on concanavalin A–Sepharose and AMP–Sepharose. The contribution to the total of soluble enzyme activity was lower in electric organ (1.6%) than in bovine cerebral cortex (27.9%). Membrane-bound and soluble forms have very similar K_m values for AMP and are inhibited by micromolar concentrations of ATP. Both forms cross-react with, and are inhibited by, an antibody against the membrane-bound surface-located (ecto-) 5′-nucleotidase from electric organ. The HNK-1 carbohydrate epitope is present on both forms of the *Torpedo* enzyme, but is entirely absent from bovine cerebral-cortex 5′-nucleotidase. An antibody specific for the inositol 1,2-(cyclic)monophosphate that is formed on phospholipase C cleavage of an intact glycosyl-phosphatidylinositol (GPI) anchor binds to the soluble, but not to the membrane-bound, form of the enzyme from both sources. Our results suggest that soluble low-K_m 5′-nucleotidase in both electric organ and bovine brain is derived from the membrane-bound GPI-anchored form of the enzyme by the action of a phospholipase C and is not a soluble cytoplasmic enzyme.

INTRODUCTION

5′-Nucleotidase (EC 3.1.3.5) activity is found in a membrane-bound form as well as in soluble forms. The membrane-bound form is glycosyl-phosphatidylinositol (GPI)-anchored and thus belongs to the increasing number of surface-located proteins of varying functional specificity which are found to be lipid-linked by GPI (Low, 1990). The K_m for AMP of ecto-5′-nucleotidase is in the low micromolar range and the enzyme is inhibited by micromolar concentrations of ADP and ATP. The lectin concanavalin A binds the enzyme and inhibits enzyme activity [see Volknandt *et al.* (1991) and references cited therein]. *Torpedo* ecto-5′-nucleotidase carries the HNK-1 carbohydrate epitope (Vogel *et al.*, 1991). This epitope is present in a variety of other surface-located proteins known to be involved in cell adhesion, such as N-CAM, L1 or cytotactin.

Soluble 5′-nucleotidase is less clearly defined. In vertebrate tissues three forms of soluble 5′-nucleotidase may be differentiated. One has a preference for IMP as a substrate and is activated by both ADP and ATP, one prefers AMP and is activated by ADP only, and a third form also prefers AMP but it is inhibited by micromolar concentrations of ADP and ATP. Whereas the first two forms display high K_m values for AMP, that of the third form is in the low micromolar range [see Volknandt *et al.* (1991) and references cited therein]. At present it is not clear whether all soluble forms occur in all tissues and cellular systems.

Many of the GPI-anchored proteins are also found in soluble form. These include, for example, N-CAM, Thy-1 or the FcIII protein of neutrophils (Low, 1990). Whereas soluble forms might originate from constitutive secretion, the possibility of cleavage of the anchor by endogenous phospholipases needs to be considered. There is evidence that the low-K_m soluble and ATP-inhibited form of 5′-nucleotidase might not be a true cytoplasmic intracellular enzyme but, rather, may be derived from a GPI-anchored precursor. GPI-anchored 5′-nucleotidase can be partially solubilized by application of GPI-specific phospholipase C (e.g. Grondal & Zimmermann, 1987; Tanaka *et al.*, 1989; Stochaj *et al.*, 1989; Lisanti *et al.*, 1989; Thompson *et al.*, 1990; Torres *et al.*, 1990), and the soluble form of 5′-nucleotidase from human placenta has been shown to contain inositol (Klemens *et al.*, 1990). Antibodies against the surface-located 5′-nucleotidase recognize or even inhibit soluble low-K_m 5′-nucleotidase. The molecular masses are similar to, or identical with, that of the ectoenzyme, and the enzyme binds the lectin concanavalin A (Zekri *et al.*, 1988; Stochaj *et al.*, 1989; Piec & Le Hir, 1991).

Liberation of GPI-anchored proteins with phospholipase C results in the formation of an 1,2-(cyclic)monophosphate on the inositol ring, the cross-reacting determinant (CRD), which can be specifically recognized by a polyclonal antibody (Hooper *et al.*, 1991). We have used this antibody to demonstrate that soluble 5′-nucleotidase isolated from bovine brain and *Torpedo* electric organ was derived from a GPI-anchored form by phospholipase C cleavage. This soluble enzyme shares the principal characteristics of the membrane-bound enzyme and is recognized and inhibited by antibodies against ecto-5′-nucleotidase. Furthermore, like the membrane-bound enzyme (Vogel *et*

Abbreviations used: CRD, cross-reacting determinant; GPI, glycosyl-phosphatidylinositol.

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al., 1991), soluble 5′-nucleotidase from electric-ray electric organ carries the HNK-1 epitope.

EXPERIMENTAL

Enzyme purification

Membrane-bound 5′-nucleotidase was isolated from the electric organ of the electric ray (*Torpedo marmorata*) as described by Grondal & Zimmermann (1987). The isolation from bovine cerebral cortex was carried out essentially as described by Volknandt *et al.* (1991), except that sulphobetaine-14 (1 %) was used for solubilization. Bovine brain was taken immediately after the animal had been stunned and homogenized after transport on ice to the laboratory (1 h) or immediately after excision in the slaughterhouse. Electric organ stored in liquid nitrogen was thawed in the coldroom to 4 °C and subsequently homogenized.

For isolation of soluble 5′-nucleotidase, tissue was homogenized by using a Waring Blendor as well as a Potter–Elvehjem homogenizer in ice-cold 20 mM-Hepes buffer, pH 7.4 (10 % w/v). After centrifugation of the homogenate (30000 $g_{av.}$, 60 min) the supernatant was re-centrifuged at 200000 $g_{av.}$ for 60 min. The resulting supernatant fraction was precipitated by addition of solid $(NH_4)SO_4$ to a final concentration of 45 % within 2–3 h and with continuous stirring on ice. After an additional 1–2 h the mixture was centrifuged at 30000 $g_{av.}$ for 30 min. The pellet was dissolved in 30 ml of buffer (20 mM-Tris/HCl/1 mM-$MgCl_2$/1 mM-$MnCl_2$/1 mM-$CaCl_2$, pH 7.4) and dialysed for 1–2 days with several changes of buffer. In part of the experiments EGTA and EDTA (1 mM each) were added during the initial homogenization step.

Affinity-chromatography steps were identical for soluble and membrane-bound enzyme fractions. Chromatography on concanavalin A–Sepharose was followed by chromatography on AMP–Sepharose as described by Grondal & Zimmermann (1987). For kinetic analysis it was essential to dialyse extensively the eluate of the AMP–Sepharose column (10 % glycerol/10 mM-Hepes, pH 7.4).

In order to estimate the relative contribution of soluble 5′-nucleotidase to the total activity, membrane-bound and soluble forms were isolated in parallel from the same piece of tissue. Enzyme activity was compared after chromatography on AMP–Sepharose. Since total high-speed supernatants contain a variety of enzyme activities capable of hydrolysing AMP, we made no attempt to compare enzyme activities at that level.

Analytical procedures

Activity of 5′-nucleotidase was determined by analysis of phosphate (Lanzetta *et al.*, 1979) using AMP (0.5 mM) in the presence of Mg^{2+} (4 mM) as a substrate. For determination of non-specific phosphatase activity, *p*-nitrophenyl phosphate (0.5 mM) was used as a substrate instead of AMP. For studying the inhibitory effect of an IgG fraction derived from a rabbit antiserum directed against *Torpedo* electric-organ 5′-nucleotidase, the enzyme purified from either electric organ or bovine cerebral cortex was preincubated for 0.5 h as previously described (Grondal & Zimmermann, 1987). Protein was determined by the method of Peterson (1977), with BSA as a standard.

Electrophoretic techniques and immunodetection

SDS/PAGE (Laemmli, 1970) was carried out on Minigels (thickness 1 mm) with acrylamide concentrations of 5 % (w/v) for the stacking gel and 10 % (w/v) for the running gel. Molecular masses were determined using standard proteins (Sigma, Merck). After transfer to nitrocellulose (Towbin *et al.*, 1979) immunodetection was performed using iodinated or horseradish-peroxidase-conjugated second antibodies (Volknandt *et al.*, 1987). 5′-Nucleotidase and the HNK-1 epitope were detected with a polyclonal antibody directed against *Torpedo* electric-organ ecto-5′-nucleotidase (Grondal & Zimmermann, 1987) and a monoclonal antibody (Vogel *et al.*, 1991) respectively. The anti-5′-nucleotidase antibody had previously been shown (Volknandt *et al.*, 1991) to recognize membrane-bound 5′-nucleotidase also from mammalian sources. The CRD on the GPI-anchor was identified using a polyclonal antibody (RP147) produced against phospholipase C-solubilized pig membrane dipeptidase that recognizes the inositol 1,2-(cyclic)monophosphate formed after cleavage of the intact GPI anchor with phospholipase C (Hooper *et al.*, 1991). In order to investigate the specificity of antibody binding, the soluble form of 5′-nucleotidase was subjected to mild acid treatment to cleave the inositol 1,2-(cyclic)phosphate ring (Hooper *et al.*, 1991). In brief, the eluate fraction derived from the AMP-affinity column was treated with HCl (1 M) for 30 min (room temperature). After adjustment of the pH to 7.4 with NaOH, protein was precipitated with a 5-fold volume of acetone (−20 °C). After 5 min at −20 °C the sample was centrifuged (10 min, 15000 $g_{av.}$). The pellet was taken up in SDS-sample buffer and processed for SDS/PAGE.

RESULTS

Soluble 5′-nucleotidase is defined as the enzyme activity that remains in the supernatant fraction after high-speed centrifugation (200000 $g_{av.}$ and is precipitated by 45 % (w/v) $(NH_4)_2SO_4$. Precipitation was followed by affinity chromatography on concanavalin A–Sepharose and AMP–Sepharose. Thus only glycosylated 5′-nucleotidase has been the subject of our analysis. It is noteworthy that the protein fraction that did not bind to either concanavalin A or AMP did not contain significant activity for hydrolysis of either AMP or IMP. Neither membrane-bound nor soluble 5′-nucleotidase revealed activity for the hydrolysis of *p*-nitrophenyl phosphate.

In several experiments the relative proportion of soluble enzyme activity to the total was estimated. Soluble 5′-nucleotidase activity was low in homogenates from the *Torpedo* electric organ [1.6 % ± 1.2 (S.D.), $n = 3$] as compared with bovine cerebral-cortex homogenates [27.9 ± 3.8 % (range), $n = 2$]. Addition of EDTA (1 mM) and EGTA (1 mM) to the homogenization medium had either no (*Torpedo*) or only a small [bovine brain, additional 6.9 ± 2.2 % (range), $n = 2$] effect on the contribution of soluble enzyme activity. Nor did the length of the thawing period (up to 10 h at 4 °C) of previously frozen electric organ significantly influence the contribution of soluble enzyme activity. We did find, however, that the contribution of soluble enzyme activity increases with time *post mortem* in bovine cerebral cortex [from 27.9 % when the cortex was homogenized in the slaughterhouse immediately after excision of the brain to 70.4 ± 6.0 % (range), $n = 2$, after approx. 1 h transport of the brain on ice to the laboratory].

K_m values for AMP are identical (bovine cerebral cortex) or very similar (electric organ) for isolated membrane-bound and soluble 5′-nucleotidases and range from 26 to 45 μM (Fig. 1). In either case micromolar concentrations of ATP are inhibitory to enzyme activity.

On SDS/PAGE, 5′-nucleotidase from all sources investigated showed an apparent molecular mass of approx. 62 kDa (Fig. 2). Both membrane-bound and soluble 5′-nucleotidase bind the monospecific anti-ecto-5′-nucleotidase antibody. Western blots reveal a tendency of 5′-nucleotidase from *Torpedo* electric organ to form a double band at 60 and 62 kDa (Fig. 2). The lower band is fainter. A double band is prominent in preparations from bovine cerebral cortex. Interestingly, both membrane-bound and soluble 5′-nucleotidases from electric organ are recognized by the

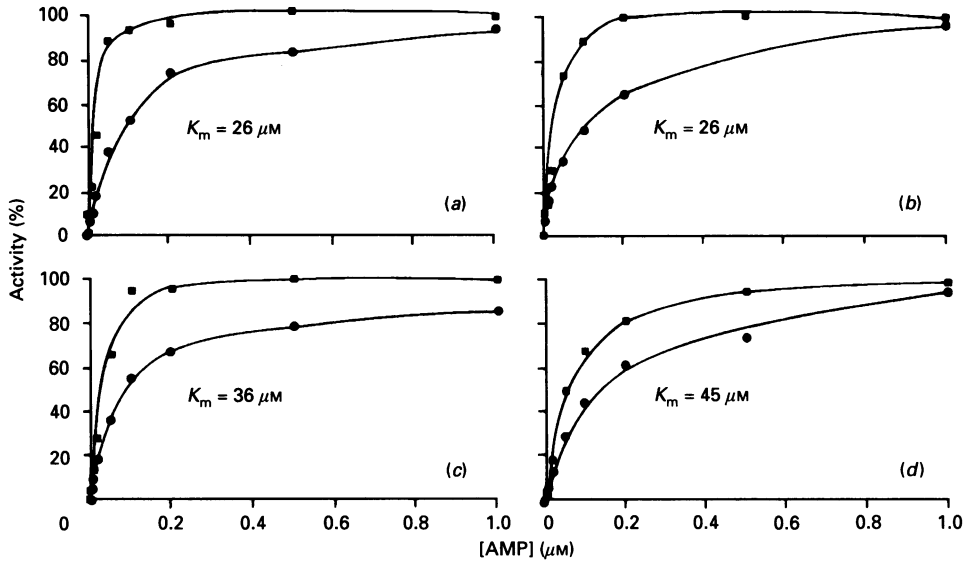

Fig. 1. Comparison of principal kinetic characteristics of soluble and membrane-bound low-K_m-5′-nucleotidase

Velocity (standardized to 100%) was measured at various concentrations of AMP in the absence (■) or presence (●) of 200 μM-ATP. Sources of isolated 5′-nucleotidase and maximal velocities (nmol of P_i/min per mg of protein, in parentheses) were: (*a*) bovine cerebral cortex, soluble (47); (*b*) bovine cerebral cortex, membrane-bound (34); (*c*) electric organ, soluble (36); (*d*) electric organ, membrane-bound (60).

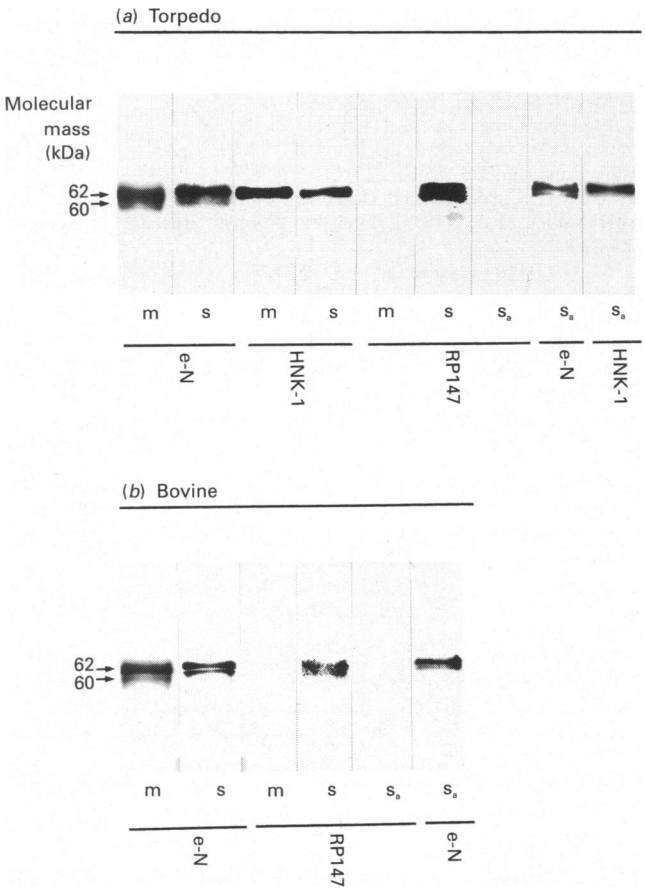

Fig. 2. Antibody-binding characteristics of isolated membrane-bound and soluble 5′-nucleotidase as demonstrated in Western blots

(*a*) *Torpedo* electric organ; (*b*) bovine cerebral cortex. Fractions analysed represent membrane-bound (m), soluble (s) or previously acid-treated soluble (s_a) enzyme. Antibodies applied were anti-ecto-5′-nucleotidase antibody (e-N), HNK-1 antibody (HNK-1) and antibody RP147 (RP147). Equal amounts of protein (1.5 μg) were loaded per lane.

monoclonal anti-HNK-1 antibody (Figs. 2*c* and 2*d*). It is, however, only the upper band at 62 kDa that is HNK-1-immunoreactive. In contrast, bovine cerebral cortex 5′-nucleotidase is entirely HNK-1-negative (results not shown).

Membrane-bound forms of 5′-nucleotidase from either *Torpedo* electric organ or bovine brain do not bind the RP147 antibody, which is expected to recognize the 1,2-(cyclic)monophosphate on the inositol ring of the cleaved GPI anchor (Fig. 2). However, the soluble enzyme forms from both sources were recognized by the RP147 antibody. The broad bands in the Western blots suggest that the antibody binds to each band within the doublet. If isolated soluble 5′-nucleotidase is treated with mild acid to hydrolyse the cyclic monophosphate diester bond, the RP147 antibody no longer recognizes the enzyme. However, the immunoreactivity towards the anti-ecto-5′-nucleotidase antibody and the HNK-1 antibody (*Torpedo* only) is still preserved.

The anti-(*Torpedo*-electric-organ ecto-5′-nucleotidase) antibody maximally inhibited the membrane-bound form of electric-organ-derived 5′-nucleotidase by 95±1.3% and the soluble form by 93±1.1%. The same IgG fraction inhibited membrane-bound 5′-nucleotidase from bovine cerebral cortex by 50±2.2% and the soluble form by 40±2.1% (all values ±S.D., $n = 3$).

DISCUSSION

In GPI-anchored ecto-5′-nucleotidase of rat liver and human placenta, the *C*-terminal amino acid (serine-523) is covalently attached via phosphoethanolamine to a sequence of three mannose residues. The reducing end of the mannose chain is in turn linked via glucosamine to the inositol head-group of a phosphatidylinositol molecule (Misumi *et al.*, 1990*b*; Ogata *et al.*, 1990). As for rat liver and human placenta, the *C*-terminal amino acid sequence deduced from the cDNA encoding electric-ray 5′-nucleotidase also contains a serine in position 523 followed by a stretch of hydrophobic amino acids (Volknandt *et al.*, 1991). This, together with the observation that the enzyme can at least in part be solubilized by GPI-specific phospholipase C (Grondal & Zimmermann, 1987), suggests that the electric-ray enzyme also carries a GPI anchor. The GPI anchor of the membrane-bound

form of 5′-nucleotidase could be acted on by phospholipases, resulting in the release of soluble enzyme. A phospholipase D has been purified to homogeneity from blood plasma (Davitz *et al.*, 1989, Huang *et al.*, 1990) which is speculated to have a role in cleaving GPI anchors, and a GPI-specific phospholipase D activity has been partially purified from bovine brain (Hoener *et al.*, 1990). However, a phospholipase D would hydrolyse the inositol phosphate bond, generating a soluble form of the protein which would lack the inositol 1,2-(cyclic)monophosphate epitope and would thus not cross-react with the anti-CRD antibody (Hooper & Turner, 1989). Soluble 5′-nucleotidase from both bovine cerebral cortex and *Torpedo* electric organ is specifically recognized by the anti-CRD antibody, indicating that they are the result of a phospholipase C cleavage. A GPI-specific phospholipase C has been described in rat liver membranes (Fox *et al.*, 1987), and the GPI-anchored pancreatic-granule membrane protein GP-2 is also solubilized by an endogenous phospholipase C, with concomitant generation of the inositol 1,2-(cyclic)monophosphate epitope (Paul *et al.*, 1991). Thus it is tempting to speculate that an endogenous phospholipase C could release 5′-nucleotidase from its lipid anchor under controlled conditions.

The presence of low-K_m soluble forms of 5′-nucleotidase which bear similarities to the form we have studied has been described in a variety of tissues, including bovine brain (Montero & Fes, 1982; Mallol & Bozal, 1983) and rat brain (Lai & Wong, 1991; Orford *et al.*, 1991). In rat liver (Fritzson *et al.*, 1986) and kidney (Piec & Le Hir, 1991) the contribution to the total of the low K_m soluble form is in the range of 1–7% and similar to that observed for the electric-ray electric organ. In contrast, the contribution in human placenta is considerably higher (25%; Klemens *et al.*, 1990) and in brain has been estimated to be 30% (Lai & Wong, 1991) or even 94.5% (Montero & Fes, 1982). There may be tissue-specific differences in the degree of soluble low-K_m 5′-nucleotidase, and this may correlate with the effectiveness of 5′-nucleotidase release by GPI-specific phospholipase C *in vitro* (Grondal & Zimmermann, 1987; Stochaj *et al.*, 1989). Although our results suggest that, at least in mammalian brain, the contribution of the soluble form increases with time *post mortem*, and the high contribution (28%) of the soluble form after immediate homogenization would suggest that it does not entirely represent an artifact of preparation.

The general kinetic characteristics of the soluble form, as well as the recognition by anti-ecto-5′-nucleotidase antibodies, also suggest a close relationship to the surface-located GPI-anchored enzyme. This is further supported by its glycosylation (Klemens *et al.*, 1990; Piec & Le Hir, 1991; the present study), and our observation that the soluble, like the membrane-anchored, enzyme from the electric ray carries the HNK-1 carbohydrate epitope. At present the appearance of a doublet in Western blots of both soluble and membrane-bound forms of the enzyme is not clear. To date there is no evidence for the presence of more than one ecto-5′-nucleotidase gene in one animal or for splicing variants (Volknandt *et al.*, 1991), although it has been speculated that a second form of ecto-5′-nucleotidase with a conventional transmembrane anchor might exist (Klemens *et al.*, 1990). Although we cannot exclude the possibility of proteolytic truncation during preparation, the observation that, in *Torpedo*, only the 62 kDa component carries the HNK-1 carbohydrate epitope, would support the notion of differing glycosylation. Both components, however, carry the GPI anchor.

It is noteworthy in this connection that human seminal plasma (Fini *et al.*, 1991), as well as synovial fluid (Wortmann *et al.*, 1991), contains soluble 5′-nucleotidase. Levels in the eye vary with clinical syndromes. A release of 5′-nucleotidase into the extracellular space would increase the functional capabilities of the protein, which is thought to function not only as an enzyme but to have also the potential to act in cell–cell and cell–matrix interactions (Vogel *et al.*, 1991). Although the plasma membrane is the most likely source of soluble low-K_m-5′-nucleotidase, other sources need to be considered. Intracellular vesicles can contain the enzyme, and phase-separation experiments with 5′-nucleotidase from liver lysosomes showed that 25% of the lysosomal enzyme is not GPI-anchored, but soluble (Tanaka *et al.*, 1989).

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